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A novel autophosphorylation mediated regulation of nitrite reductase in *Candida utilis*

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Abstract The assimilatory nitrite reductase catalyses the conversion of nitrite to ammonia. The enzyme from *Candida utilis* has been previously purified to homogeneity and shown to be a heterodimer consisting of 58 kDa and 66 kDa subunits. The enzyme has also been shown to be induced by nitrate and repressed by ammonium ions. The levels of nitrite reductase mRNA, its protein and the enzyme activity were modulated together indicating that the primary level of regulation of this enzyme existed at the transcriptional level. Here we report that the 58 kDa and 66 kDa subunits of the enzyme were differentially phosphorylated under the induced and repressed conditions, indicating a second level of regulation. The highly phosphorylated 66 kDa subunit was shown to be dephosphorylated by calf intestinal alkaline phosphatase. The enzymatic activity associated with the native enzyme also decreased due to the dephosphorylation. Each of the subunits could undergo autophosphorylation at serine/threonine residues as demonstrated by thin layer chromatography and recognition by antibodies to phosphoamino acids. The presence of similar phosphorylated subunits under in vivo conditions has also been demonstrated. A model has been proposed to explain the post-translational regulation of the enzyme.

Key words: Nitrite reductase; Regulation; Post-translational control; Phosphorylation; *Candida utilis*

1. Introduction

Nitrite reductase is one of the key enzymes in the nitrogen assimilation pathway, involved in the conversion of nitrite to ammonia. Although the purification and characterisation of nitrite reductases from many systems have been studied in great detail, the regulation of the enzyme is a comparatively less studied aspect. The gene expression is controlled predominantly at the level of transcript accumulation. It has been found in many cases that the induction of gene expression is caused by nitrate or nitrite and the repression by ammonia. Nitrate has been shown to induce nitrite reductase activity in maize [1,2], spinach [3], *Aspergillus nidulans* [4], barley [5] and mustard [6]. Apart from plants, the transcriptional control for nitrite reductase gene expression has been studied in cyanobacteria [7] and *Chlamydomonas reinhardtii* [8]. In *Neurospora crassa* [9] and the yeast *Hansenula wingei* [10], the regulation has been studied only at the level of enzymatic activity. A few cases of post-translational modifications of nitrite reductase have been reported from various systems [11–13]. However, the regulation of this enzyme due to post-translational modifications has not yet been reported.

Candida utilis, a unicellular eukaryote, belongs to the yeast family which is universally recognised as an important model system for the study of genetics. Apart from its many industrial uses, *C. utilis* serves as an ideal system for the study of nitrogen assimilation by virtue of its rapid growth, non-pathogenicity and adaptability to grow on a single inorganic source of nitrogen [14]. Phosphorylated enzymes have been characterised from this organism for quite some time [15–18]. Recently a phosphorylation dependent signal transduction pathway has been implicated in the activation of cytoplasmic trehalese in *C. utilis* [19].

We have previously purified and characterised the nitrite reductase from *C. utilis* and shown it to be a heterodimer consisting of 66 kDa and 58 kDa subunits. The phosphorylated nature of the 66 kDa subunit was demonstrated when the enzyme was purified with nitrate as the source of nitrogen [20]. We have also recently shown that the primary level of regulation for this enzyme was transcriptional and consequently the effect was also reflected at the translational level [21]. Here we report that a second level of regulation exists for this enzyme at the post-translational stage in the form of differential phosphorylation of the subunits under induced and repressed conditions. A model has been proposed to explain the mechanism by which nitrite reductase is regulated in *C. utilis*.

2. Materials and methods

2.1. Materials

The ECL chemiluminescence kit and calf intestinal alkaline phosphatase (CIAP) were obtained from Amersham International, Amersham, Bucks, UK. [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol) and [^{32}P]orthophosphate (carrier free) were purchased from Bhabha Atomic Research Centre, Bombay, India. X-ray film XR-2, developer and fixer were obtained from Indian Photographic Company Limited, Bombay, India. Zymolase, adenosine triphosphate (ATP), dithiothreitol, phosphoamino acids, phosphoserine and phosphotyrosine antibodies were purchased from Sigma Chemical Company, St. Louis, MO, USA. Yeast extract and peptone were purchased from Difco Laboratories, Detroit, MI, USA. Cellulose coated thin layer chromatographic (TLC) plates were bought from E. Merck, Darmstadt, Germany. All other chemicals of analytical grade were obtained from BDH Limited, Bombay, India or from Sarabhai Chemicals, Baroda, India.

2.2. Organism

C. utilis CBS 4511 (wild type), obtained from Centraalbureau voor Schimmelcultures, Delft, The Netherlands, was used in all experiments.

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2.3. Maintenance and growth conditions

The *C. utilis* cultures were maintained by monthly transfers on Sabouraud's dextrose agar slants containing 1% neopeptone, 1% dextrose, 0.2% yeast extract and 2% agar.

The basal medium used to grow *C. utilis* was essentially as described by Wickerham [22]. Nitrate and ammonia were used respectively as supplements for the induction and repression of the enzyme as determined previously [21].

2.4. Preparation of cell free extract

The harvested cells were resuspended in Tris-HCl buffer (10 mM, pH 8) containing 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM 2-mercaptoethanol and 10% glycerol and disrupted in a French pressure cell at 15000 psi twice. The slurry was centrifuged at $8000 \times g$ for 20 min at 4°C and the supernatant was used for further experiments.

2.5. Purification and assay of nitrite reductase

The cells were grown with glucose (1%) as the sole source of carbon, and nitrate (50 mM) as the sole source of nitrogen. The enzyme was purified to homogeneity as described earlier [20]. Methyl viologen nitrite reductase activity was assayed by the method of Ida [23]. Nitrite disappearance was determined after a 100-fold dilution of the reaction mixture by the diazo coupling method [24].

2.6. Estimation of proteins

The protein content was determined by the method of Lowry as modified by Hartree [25] using crystalline bovine serum albumin as standard.

2.7. Electrophoresis and western immunoblot analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [26]. Western blot analysis was carried out essentially according to the method of Towbin et al. [27]. For the phosphoamino acid analysis of the nitrite reductase subunits, phosphoserine and phosphotyrosine antibodies were used at a dilution of 1:12500. The corresponding secondary antibody from the chemiluminescence kit was used at a dilution of 1:5000. All detections were done according to the supplier's protocol.

2.8. Immunoprecipitation

Immunoprecipitations were performed with crude cell lysates, according to the method of Perbal [28]. The polyclonal antibody against *Cucurbita pepo* nitrite reductase [29] was used at a dilution of 1:2000.

2.9. Separation and renaturation of subunits

The two subunits of nitrite reductase were eluted out of the SDS-PAGE gel and renatured according to the method of Hager and Burgess [30].

2.10. Assay for phosphatase activity

The phosphatase activity of the separated subunits, reconstituted enzyme, and the native enzyme was assayed using *p*-nitrophenylphosphate as the substrate [31].

2.11. Assay for protein kinase activity

The assay for the protein kinase activity was carried out according to Lorberboum et al. [32]. The subunits, individually (20 μg protein) or together (10 μg of each of the subunits), were incubated with [γ-^{32}P]ATP at 30°C for 30 min. The presence of phosphorylated products were determined either by estimating the TCA insoluble Cerenkov counts in a scintillation spectrometer or by subjecting a portion of the reaction mix to SDS-PAGE and subsequent autoradiography of the dried gel.

2.12. Phosphoamino acid analysis by thin layer chromatography

The radiolabelled subunits of nitrite reductase (obtained by incubating 10 μg of each subunit with 20 μCi of [γ-^{32}P]ATP) were hydrolysed with 6 N HCl in a vacuum sealed tube for 1 h and phosphoamino acid analysis was carried out according to Boyle et al. [33].

3. Results

3.1. Differential phosphorylation of the subunits under induced (nitrate grown) and repressed (ammonium grown) conditions

The *C. utilis* cells grown in the presence of either nitrate or ammonia were labelled with [^{32}P]orthophosphate and cell extracts (having equal amounts of protein) were immunoprecipitated with *Cucurbita pepo* nitrite reductase antibody under the same experimental conditions. A differential phosphorylation pattern of the two subunits of nitrite reductase was reproducibly obtained. When grown in presence of nitrate, only the 66 kDa subunit was phosphorylated, while both 58 kDa and 66 kDa subunits were phosphorylated when ammonium salt was used as the source of nitrogen (Fig. 1A).

Quantitation of the phosphorylated subunits under the different conditions indicated that the 66 kDa subunit was phosphorylated at least 3-fold more under the nitrate grown conditions. On the other hand, the 58 kDa subunit underwent a 5–6-fold hyper-phosphorylation under the ammonium grown condition compared to the nitrate grown one where its phosphorylation status was negligible. However, the extent of the 58 kDa phosphorylation was still much less (3–4-fold) when compared to the 66 kDa phosphorylation under the induced conditions (Fig. 1B).

3.2. Effect of phosphatase on nitrite reductase and its subunits

Since the 66 kDa subunit was found to be highly phosphorylated under induced (nitrate grown) condition while being phosphorylated to a much lower extent under the repressed (ammonium salt grown) condition, the possibility was there that either the 58 kDa or the 66 kDa subunit may have possessed phosphatase activity leading to dephosphorylation of the subunits. To examine this aspect purified and renatured 58 kDa and 66 kDa subunits were incubated with *p*-nitrophenylphosphate and the release of *p*-nitrophenol was monitored. However, attempts to detect phosphatase activity associated with both the subunits, either individually or in combination, were unsuccessful (data not shown).

CIAP, tested to be free of any protease activity (data not shown), was chosen as a prototype phosphatase in further studies. CIAP was employed to dephosphorylate the purified labelled 66 kDa subunit of nitrite reductase. Most of the labelled phosphates could be removed within a 30 min interval (Fig. 2A, lanes 2–5). The fact that the phosphate groups on the 66 kDa subunit were accessible to phosphatase was further proved when the incubation of the subunit with CIAP was carried out in presence of nitrilotriacetic acid, a CIAP inhibitor. Even after a 30 min incubation there was no loss in the amount of radioactivity attached with the subunit (Fig. 2A, lane 1).

Nitrite reductase, purified from cells grown under induced conditions was incubated with CIAP for various time intervals. It was observed that the activity of the enzyme decreases with the increase in duration of incubation with CIAP compared to the control experiments where the incubation was carried out at the same time intervals but in the absence of CIAP. The loss in enzyme activity was around 80% within 30 min of incubation (Fig. 2B).

3.3. Autophosphorylation of the subunits of nitrite reductase

To determine whether the subunits of nitrite reductase had

the capability of autophosphorylation, the individual subunits were incubated with [γ-^{32}P]ATP (10 μCi) in kinase buffer. Though the radioactivity associated with the acid-insoluble material indicated a low autophosphorylation level of both 58 kDa and 66 kDa subunits (Fig. 3A, A and B), the phosphorylated products were not detected on autoradiography (Fig. 3B, lanes 1 and 2). Therefore the experiment was repeated with higher [γ-^{32}P]ATP amounts (40 μCi). When the reaction mixture was subjected to SDS-PAGE and the subsequent gel autoradiographed, very low levels of the phosphorylated products were visible (Fig. 3C, lanes 1 and 2).

The 66 kDa subunit when dephosphorylated in vitro with CIAP proved to be a better substrate for the addition of new phosphates and undergoes rephosphorylation to a very high extent, as shown in Fig. 3B, lane 3. So the possibilities that existed were: (i) the 58 kDa subunit had the ability to phosphorylate the other subunit by phosphotransfer, (ii) the larger (66 kDa) subunit can autophosphorylate itself in spite of the presence of the smaller subunit (58 kDa). To determine which one of the above possibilities was actually happening, the 58 kDa subunit was incubated with the 66 kDa subunit, in native and dephosphorylated states, in presence of [γ-^{32}P]ATP (10 μCi). A phosphorylated product was obtained when 58 kDa subunit was incubated with dephosphorylated 66 kDa (Fig. 3B, lane 4) and not when incubated with normal phosphorylated 66 kDa subunit (Fig. 3B, lane 5). This showed that autophosphorylation to be an intrinsic property of the 66 kDa subunit and not due to the kinase activity of the 58 kDa subunit on the 66 kDa subunit.

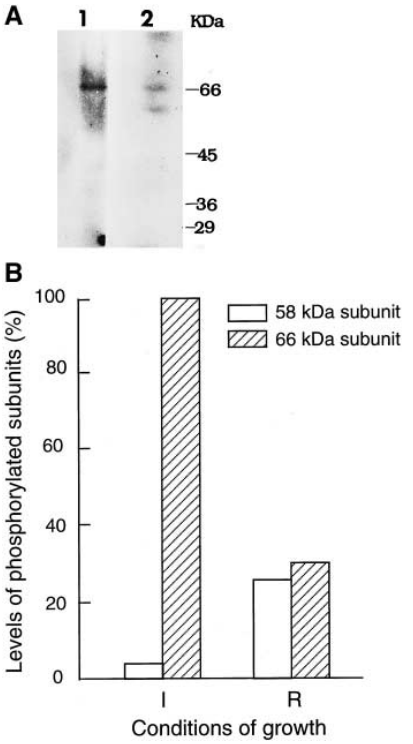

Fig. 1. Phosphorylation of the subunits of nitrite reductase. A: Differential phosphorylation status. The *C. utilis* cells were grown under the condition of induction (nitrate) (lane 1) or repression (ammonium ions) (lane 2) in phosphate free Wickerham medium but in the presence of [^{32}P]orthophosphate. Aliquots of the cell free extracts (containing 200 μg of protein) were immunoprecipitated (as described in Section 2), and the immunoprecipitates were subjected to 10% SDS-PAGE and the gel was subsequently autoradiographed. B: Quantitation of the phosphorylation of the subunits. The autoradiograph shown in A was quantitated in a LKB 2202 Ultroscan Laser Densitometer. The two different conditions of growth are indicated as induction (I) and repression (R). The experiment was carried out thrice and the quantitations were reproducible. The phosphorylation of the 66 kDa subunit under induced condition (I) was considered as 100% and relative to it the levels were calculated for other subunits.

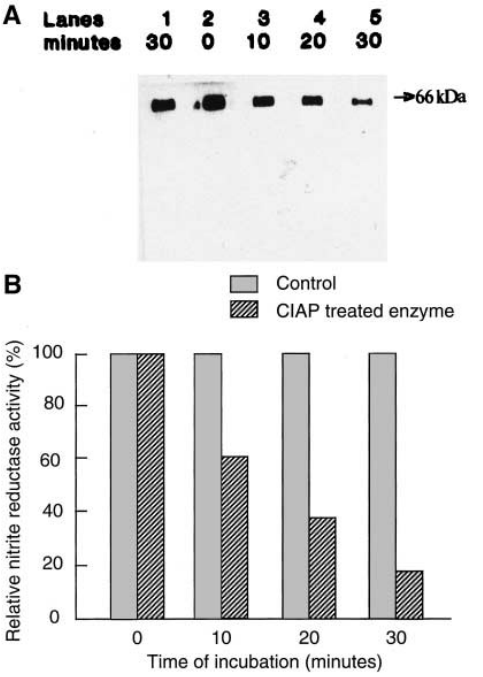

Fig. 2. Effect of calf intestinal alkaline phosphatase. A: Dephosphorylation of 66 kDa subunit. The 66 kDa subunit which was labelled under induced conditions was eluted from the gel, renatured and incubated at different time intervals with 0.5 units of CIAP. The reaction mix was subjected to 10% SDS-PAGE. Lane 1: 66 kDa (20 μg protein) treated with CIAP for 30 min along with nitrilotriacetic acid (20 mM, final concentration); lanes 2–5: 66 kDa (20 μg protein in each case) treated with CIAP for different time intervals. The gel was subsequently autoradiographed. B: Effect on nitrite reductase activity. The purified native enzyme (20 μg protein) was incubated with 0.5 units of CIAP at 30°C. The reactions were stopped at definite time intervals and nitrite reductase activity was assayed immediately. In control experiments where CIAP was not added assays were carried out after the preincubation of the enzyme at the same time intervals. The nitrite reductase activity in absence of CIAP at the beginning of the incubation (i.e. at zero time point) was considered as 100% and the values obtained for both the control and CIAP treated enzyme (at different time intervals) were calculated relative to it.

However, there still remained the possibility that the phosphorylated products seen were the result of limited autophosphorylating ability of the 58 kDa subunit. To check this possibility, the 58 kDa subunit was preincubated with unlabelled ATP (Fig. 3B, lanes 6 and 7), so that its autophosphorylation takes place prior to its incubation with native or dephosphorylated 66 kDa subunit in presence of 40 μCi of [γ-^{32}P]ATP. The presence of the same phosphorylated products again proved the different extents of the autophosphorylating abilities of the 66 kDa subunit in the phosphorylated and dephosphorylated conditions. During the above set of experiments several positive controls (proteins reported in literature to autophosphorylate) were tested and a negative control was always included (data not shown). This was carried

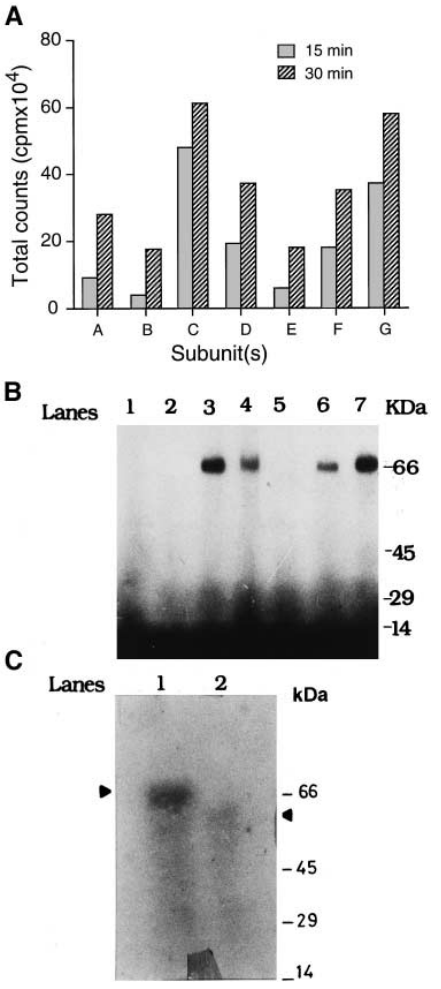

Fig. 3. Autophosphorylation of the subunits. A: Cerenkov counts in scintillation spectrometer. The purified phosphorylated 66 kDa subunit was dephosphorylated for 30 min by CIAP, the dephosphorylated subunit subsequently subjected to 10% SDS-PAGE, eluted and renatured to free itself from the phosphatase. The autophosphorylation of the subunits, separately (20 μg of protein), or in combination (10 μg of each of the subunits), was carried out in a kinase buffer using [γ-^{32}P]ATP (10 μCi for A–E and 40 μCi for F and G) as described in Section 2. Aliquots were removed after 15 and 30 min, absorbed on Whatman No. 3, processed (as detailed in Section 2) and Cerenkov counts were taken in a scintillation spectrometer. A: 66 kDa subunit; B: 58 kDa subunit; C: dephosphorylated 66 kDa subunit; D: 58 kDa subunit incubated with dephosphorylated 66 kDa subunit; E: 58 kDa subunit incubated with 66 kDa subunit; F: 58 kDa subunit preincubated with cold ATP (1 mM, final concentration) for 30 min, followed by incubation with 66 kDa subunit; G: 58 kDa subunit preincubated with cold ATP (1 mM, final concentration) for 30 min, followed by incubation with dephosphorylated 66 kDa subunit. The values indicated represent the mean values of three sets of independent experiments. B: SDS-PAGE analysis of the autophosphorylation of the subunits. The autophosphorylation of the subunits, either individually or in combination, was carried out as in A (details in Section 2). The phosphorylated products obtained after 30 min of incubation were subjected to 10% SDS-PAGE. Lane 1: 66 kDa subunit; lane 2: 58 kDa subunit; lane 3: dephosphorylated 66 kDa subunit; lane 4: 58 kDa subunit incubated with dephosphorylated 66 kDa subunit; lane 5: 58 kDa subunit incubated with 66 kDa subunit; lane 6: 58 kDa subunit preincubated with cold ATP (1 mM, final concentration) for 30 min, followed by incubation with 66 kDa subunit and lane 7: 58 kDa subunit preincubated with cold ATP (1 mM, final concentration) for 30 min followed by incubation with dephosphorylated 66 kDa subunit. The gel was subsequently autoradiographed. C: SDS-PAGE analysis of the autophosphorylation of 58 kDa and 66 kDa subunits. The autophosphorylation of the 66 kDa (lane 1) and 58 kDa subunit (lane 2) in kinase buffer using 40 μCi [γ-^{32}P]ATP was carried out as described in Section 2. The products were subjected to 10% SDS-PAGE and the gel was subsequently autoradiographed. ←

out to discount the possibility of non-specific radiolabelling of subunits, specially since a comparatively high amount of radioactivity have been used.

3.4. Phosphoamino acid analysis

The dephosphorylated 66 kDa subunit after in vitro phosphorylation with cold 1 mM ATP was subjected to immunoblotting with phosphoamino acid antibodies. A phosphorylated product of 66 kDa was observed with phosphoserine antibody as the probe (Fig. 4A, lane 1). The same product was detectable with lower intensity when the normal 66 kDa subunit was probed with the same antibody (Fig. 4A, lane 2). The dephosphorylated 66 kDa on subsequent phosphorylation with cold ATP underwent exhaustive phosphorylation which possibly accounts for the strong signal obtained with phosphoserine antibody. Since the normal 66 kDa subunit was not subjected to the treatment with cold ATP, the signal obtained with the same antibody was weaker. No products were observed when phosphotyrosine antibody (Fig. 4A, lanes 3 and 4) or normal rabbit serum (Fig. 4A, lanes 5 and 6) were used.

Since the three-dimensional conformations of serine and threonine residues are almost similar, there remained a possibility that the phosphoserine antibodies had also cross-reacted with phosphothreonine residues. The phosphoamino acid detection by antibody was confirmed by TLC, after acid hydrolysis of the radiolabelled phosphorylated products. Autoradiography of the chromatograph indicated that threonine was phosphorylated to a greater extent than serine when dephosphorylated 66 kDa subunit was used (Fig. 4B, lane A). In case of the native 66 kDa subunit, both the serine and threonine residues get phosphorylated equally but to lower levels (Fig. 4B, lane B).

Equal amounts of crude extracts of *C. utilis* cells, grown in the presence of either nitrate or ammonium, were immunoprecipitated with nitrite reductase antibody. The immunoprecipitates were subjected to SDS-PAGE followed by Western blot analysis with phosphoserine antibody to check whether under in vivo conditions the subunits were phosphorylated in the serine and threonine residues. It was found that phosphoserine antibody was able to detect the 66 kDa subunit under nitrate grown conditions (Fig. 4C, lane 1), while it was able to detect both the 58 kDa and 66 kDa subunits under the ammonium grown conditions (Fig. 4C, lane 2). The experiment also confirmed that in vivo the number of phosphorylated serine and threonine residues were much more in the 66 kDa subunit under induced conditions when compared to both the subunits under the repressed conditions.

4. Discussion

It has been previously observed by us that *C. utilis* was able to grow on a variety of nitrogen sources. Nitrate, the first compound in the pathway, induced nitrite reductase synthesis while ammonium repressed the enzyme at the transcriptional

level. In the presence of ammonium ions (repressor), a basal but significant level of nitrite reductase activity and protein was observed [21]. This gave rise to the possibility of yet another level of regulation, specially at the post-translational level. Here it has been clearly demonstrated that a differential phosphorylation of subunits of the subunits under the induced and repressed conditions did exist for the two subunits of nitrite reductase (Fig. 1). The two phosphorylated conditions corresponded with the vast difference in the enzymatic status of nitrite reductase (Fig. 2).

Regulation by differential phosphorylation could be caused by two processes – dephosphorylation and phosphorylation.

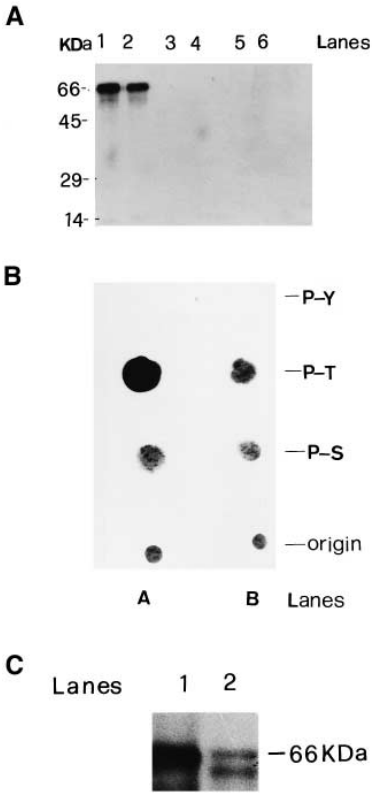

Fig. 4. Phosphoamino acid detection in the subunits. A: In vitro analysis by antibody. The dephosphorylated 66 kDa subunit was autophosphorylated using cold ATP (1 mM, final concentration). The rephosphorylated product (lanes 1, 3 and 5) was electrophoresed on 10% SDS-PAGE along with the native 66 kDa subunit (lanes 2, 4 and 6). The electrophoretically separated proteins were transferred to poly(vinylidene difluoride) membrane and the transferred proteins were subjected to Western analysis (as described in Section 2) and probed with phosphoserine antibody (lanes 1 and 2), phosphothreonine antibody (lanes 3 and 4) and normal rabbit serum (lanes 5 and 6). B: In vitro analysis by TLC. The 66 kDa subunit after dephosphorylation (lane A) and in native condition (lane B) was incubated with 40 μCi [γ-^{32}P]ATP. The labelled phosphorylated products were subjected to acid hydrolysis and separated on TLC plates. The unlabelled internal standards were detected by ninhydrin staining, while the TLC plates were autoradiographed to detect the phosphoamino acids in the samples. C: In vivo analysis by antibody. Cell extracts (200 μg in each case) from *C. utilis* grown in presence of either nitrate (lane 1) or ammonium (lane 2) were immunoprecipitated with heterologous nitrite reductase antibody (as described in Section 2) to preferentially select the enzyme molecules. The immunoprecipitates were subjected to 10% SDS-PAGE. The electrophoretically separated proteins were transferred to poly(vinylidene difluoride) membrane (details in Section 2) and Western analysis of the transferred proteins was carried out using phosphoserine antibody as the probe.

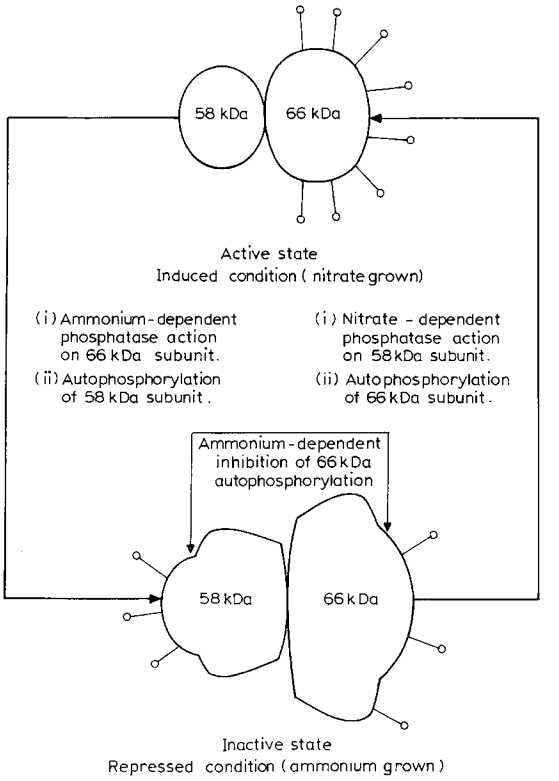

Fig. 5. A proposed model for post-translational regulation of nitrite reductase in *C. utilis*.

Each of these two processes could be regulated by an autoregulatory loop or via some transacting factors. In the case of nitrite reductase from *C. utilis* the transition from a higher phosphorylation state to a lower phosphorylation state (i.e. the dephosphorylation step) was possibly not autoregulatory as neither of the subunits, individually or in trans, showed detectable phosphatase activity. So it is probably dependent on some unknown cellular phosphatases. In fact serine-threonine phosphatases have been shown to be present in fission yeast [34,35] and filamentous fungi [36].

Regulation of gene expression by autophosphorylation have only recently become known [37–40]. In the case of nitrite reductase from *C. utilis*, it was initially shown by in vitro studies that the subunits can autophosphorylate themselves at serine and threonine residues to different extents (Figs. 3 and 4A,B). The in vivo detection of the phosphorylated serine (and threonine) residues on the differentially phosphorylated subunits of nitrite reductase indicated the biological relevance of the in vitro analysis (Fig. 4C).

So based on the evidence available it is proposed that there exists another level of regulation of the enzyme at the post-translational level by reversible protein phosphorylation. A model has been proposed for the post-translational regulation of nitrite reductase taking into account the evidence presented above (Fig. 5). Under induced (nitrate grown) conditions, the enzyme remained in an active conformation, with its 66 kDa phosphorylated (as represented by stalks) while the 58 kDa subunit remained in a non-phosphorylated condition. As soon as the repressor (ammonium ions) was added to the system, there was a down-regulation of the transcription of the enzyme. The enzyme that was present in the system possibly underwent partial degradation (possibly by ammonium ion

activated proteases) and the remaining molecules had a change in the phosphorylation status. The 58 kDa subunit underwent ammonium ion dependent low level phosphorylation at both serine and threonine residues, while the 66 kDa subunit was subjected to ammonium ion dependent dephosphorylation. So in the repressed (ammonium grown) state both the 58 kDa and the 66 kDa subunits are phosphorylated at few of the available serine and threonine residues (as represented by stalks). When inducer (nitrate) was added to the system, again there were possibly two simultaneous modifications of the nitrite reductase namely autophosphorylation of the hypophosphorylated 66 kDa subunit mostly at the threonine residues and complete dephosphorylation of the 58 kDa subunit. As the nitrite reductase gene is turned on in presence of the inducer (nitrate), newly synthesised enzyme molecules were formed which also underwent the appropriate post-translational modification.

Regulation by phosphorylation is becoming a known phenomenon in the nitrogen assimilatory pathway. Very recently a phosphorylated sensor protein has been implicated in nitrate assimilation in *Azotobacter chroococcum* [41]. The first enzyme in the nitrogen assimilation pathway, nitrate reductase, is known to be regulated by reversible phosphorylation [42,43]. The regions of nitrate reductase and the specific amino acid residues in them that are involved in the phosphorylation of this enzyme have been extensively characterised [44–47]. It had been proposed long ago that phosphorylation may be coupled to nitrite reductase activity in *Micrococcus denitrificans* [48]. Recently it has been postulated that nitrite reductase expression is possibly regulated at the post-transcriptional level by the nitrogen source in *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* [49]. Now, we have shown for the first time that a reversible autophosphorylation mediated regulation also exists for nitrite reductase, a key enzyme in the nitrogen assimilation pathway. A more interesting question now may be to find out why *C. utilis* requires a two-step regulation process – at the levels of transcription and post-translation. It is quite possible that the regulation of this enzyme by reversible phosphorylation is the key step by which the yeast adapts itself physiologically and rapidly to the changes in the growth conditions. This mode of autoregulation of the enzyme may also be true for nitrite reductases from other sources.

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References

[1] Privalle, L.S., Lahners, K.N., Mullins, M.A. and Rothstein, S. (1989) Plant Physiol. 90, 962–967.
[2] Kramer, V., Lahners, K., Back, E., Privalle, L.S. and Rothstein, S. (1989) Plant Physiol. 90, 1214–1220.
[3] Back, E., Buckhart, W., Mayor, M., Privalle, L.S. and Rothstein, S. (1988) Mol. Gen. Genet. 212, 20–26.
[4] Hawker, K.L., Montague, P. and Kinghorn, J.R. (1992) Mol. Gen. Genet. 231, 485–488.
[5] Aslam, M. and Huffaker, R.C. (1989) Plant Physiol. 91, 1152–1156.
[6] Rajshekhar, V.K. and Mohr, H. (1986) Planta 169, 594–599.
[7] Martin-Nieto, J., Herrero, A. and Flores, E. (1989) Arch. Microbiol. 151, 475–478.
[8] Galvan, A., Cordoba, F., Cardenas, J. and Fernandez, E. (1991) Biochim. Biophys. Acta 1074, 6–11.
[9] Garrett, R.H. (1972) Biochim. Biophys. Acta 264, 481–489.
[10] Jones, C.P., Wray, J.L. and Kinghorn, J.R. (1987) J. Gen. Microbiol. 133, 2767–2772.
[11] Gupta, S.C. and Beevers, L. (1987) Plant Physiol. 83, 750–754.
[12] Dalling, M.J., Tolbart, N.E. and Hageman, R.H. (1972) Biochim. Biophys. Acta 283, 505–512.
[13] Heath-Pagliuso, S., Huffaker, R.C. and Allard, R.W. (1984) Plant Physiol. 76, 353–358.
[14] La Rue, R.A. and Spencer, J.F.T. (1968) Can. J. Microbiol. 14, 79–86.
[15] Ohnishi, T., Panebianco, P. and Chance, B. (1972) Biochem. Biophys. Res. Commun. 49, 99–106.
[16] Grossman, S., Cobley, J.G. and Singer, T.P. (1974) J. Biol. Chem. 249, 3819–3826.
[17] Cobley, J.G., Singer, T.P., Beinert, H. and Grossman, S. (1975) J. Biol. Chem. 250, 211–217.
[18] Hemmings, B.A. (1978) J. Biol. Chem. 253, 5255–5258.
[19] Carrillo, D., Vicente-Soler, J., Fernandez, J., Soto, T., Cansado, J. and Gacto, M. (1995) Microbiology 141, 679–686.
[20] Sengupta, S., Shaila, M.S. and Rao, G.R. (1996) Biochem. J. 317, 147–155.
[21] Sengupta, S., Shaila, M.S. and Rao, G.R. (1997) (in press).
[22] Wickerham, L.M. (1946) J. Bacteriol. 52, 293–301.
[23] Ida, S. (1977) J. Biochem. 82, 915–918.
[24] Snell, F.D. and Snell, C.T. (1949) Nitrite. Colorimetric Methods of Analysis, D. van Nostrand Co., Princeton, NJ.
[25] Hartree, E.F. (1972) Anal. Biochem. 48, 422–427.
[26] Laemmli, U.K. (1970) Nature 227, 680–685.
[27] Towbin, H., Stachelin, T. and Gorden, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[28] Perbal, B. (1983) A Practical Guide to Molecular Cloning, John Wiley and Sons, New York.
[29] Bowsher, C.G., Emes, M.J., Commack, R. and Hucklesby, D.P. (1988) Planta 175, 334–340.
[30] Hager, D.F. and Burgess, R.R. (1980) Anal. Biochem. 109, 76–86.
[31] Galski, H., Fridovich, S.E., Weinstein, D., de Groot, N., Segal, S., Folman, R. and Hochberg, A.A. (1981) Biochem. J. 194, 857–866.
[32] Lorberboum, H., Galski, H., Schaft, C., Weinstein, D., de Groot, N. and Hochberg, A.A. (1986) Mol. Biol. Rep. 11, 29–35.
[33] Boyle, W.L., Geer, P.V.D. and Hunter, T. (1991) Methods Enzymol. 201, 110–149.
[34] Ohkura, H., Kinoshita, N., Mitayani, S., Toda, T. and Yanagida, H. (1989) Cell 57, 997–1007.
[35] Maeda, T., Tsai, A. and Saito, H. (1993) Mol. Cell. Biol. 13, 5408–5417.
[36] Dooman, J.H. and Morris, N.R. (1989) Cell 57, 987–996.
[37] Branson, J.P. and Attwood, P.V. (1997) Biochem. Soc. Trans. 25, 87S.
[38] Schulze-Muth, P., Irmler, S., Schroder, G. and Schroder, J. (1996) J. Biol. Chem. 271, 26684–26689.
[39] Yu, J.S. and Yang, S.D. (1995) Biochem. Biophys. Res. Commun. 207, 140–147.
[40] Saha, P. and Singh, M. (1995) Biochem. J. 305, 205–210.
[41] Munoz-Centeno, M.C., Pecina, A., Cejudo, F.J. and Paneque, A. (1996) FEBS Lett. 393, 7–12.
[42] MacKintosh, C. (1992) Biochim. Biophys. Acta 1137, 121–126.
[43] Chandok, M.R. and Sopory, S.K. (1996) Mol. Gen. Genet. 251, 599–608.
[44] LaBrie, S.T. and Crawford, N.M. (1994) J. Biol. Chem. 269, 14497–14501.
[45] Nussaume, L., Vincentz, M., Meyer, C., Boutin, J.P. and Caboche, M. (1995) Plant Cell 7, 611–621.
[46] Douglas, P., Morrice, N. and MacKintosh, C. (1995) FEBS Lett. 377, 113–117.
[47] Bachmann, M., Shiraishi, N., Campbell, W.H., Yoo, B.C., Harmon, A.C. and Huber, S.C. (1996) Plant Cell 8, 505–517.
[48] Sabater, F. (1966) Rev. Esp. Fisiol. 22, 1–5.
[49] Crete, P., Caboche, M. and Meyer, C. (1997) Plant J. 11, 625–634.